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The Embryonic Stem Cell Test, an *In Vitro* Embryotoxicity Test Using Two Permanent Mouse Cell Lines: 3T3 Fibroblasts and Embryonic Stem Cells

HORST SPIELMANN, INGEBORG POHL, BEATE DÖRING, MANFRED LIEBSCH, and
FERDINAND MOLDENHAUER

ABSTRACT

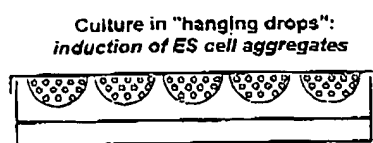
The embryonic stem cell test (EST) was developed as a new *in vitro* embryotoxicity test that does not use embryonic tissues from pregnant animals but only two permanent mouse cell lines: 3T3 fibroblasts and embryonic stem (ES) cells of the D3 line. In the EST, cytotoxicity was determined in the two cell lines for different time periods up to 10 days and, in addition, the differentiation of ES cells into contracting myocardial cells. Sixteen carefully selected test chemicals with different embryotoxic properties were tested in the EST. Of 12 endpoints and ratios of endpoints determined in the EST with the two cell lines, three endpoints were selected by stepwise discriminant analysis that showed a better correlation to the embryotoxic properties of the test chemicals than the other endpoints. Using the three endpoints and linear discriminant functions, a classification scheme was developed for the EST in which test chemicals are assigned to three classes of *in vivo* embryotoxicity: not embryotoxic, moderate and strong embryotoxic. Using this classification model all 16 test chemicals were correctly assigned in the EST to their *in vivo* classes of embryotoxicity. Such a promising result is usually not obtained in *in vitro* embryotoxicity tests, most of which are still using embryonic tissues taken from pregnant animals rather than permanent cell lines in the EST. The EST is, therefore, ready to undergo validation in other laboratories.

INTRODUCTION

Embryonic stem (ES) cells of the mouse will differentiate under appropriate culture conditions into the major embryonic tissues (Doetschmann et al., 1985; Heuer et al., 1994b). Figure 1 describes the experimental procedure for ES cell differentiation used in our laboratory. Formation of so-called embryoid bodies (EB) is

the first step of differentiation *in vitro*. It has recently been shown that EBs are fully viable and can be used to study gene expression during the early steps of embryogenesis (Gassmann et al., 1996). Several groups have used ES cells to establish *in vitro* embryotoxicity tests. In our first attempt we compared the cytotoxicity of ES cells, which served as embryonic cells, and mouse fibroblasts, representing adult

Step 1: day 0 - 3



Step 2: day 3 - 5

Cultivation in suspension culture:
differentiation into "embryoid bodies" (EBs)



Step 3: day 5 - 10

Cultivation of EBs in 24-well plates:
EBs stick to the bottom of plates and
differentiate into myocard cells

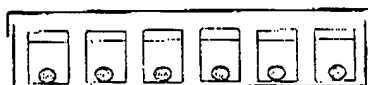


FIG. 1. Experimental model for differentiation of ES cells into contracting myocardial cells in the EST. Test chemicals are added to cell cultures on day 0 (step 1), on day 3 (step 2), and day 5 (step 3) at the same final concentration in each step. On day 10 of culture the percentage of embryoid bodies with contracting myocardial cells is determined.

cells, to assess the embryotoxic potential of test chemicals (Laschinski et al., 1991). Later we compared the cytotoxicity and inhibition of differentiation of ES cells of the cell line D3 for the same purpose (Heuer et al., 1994a, b). Quite independently Newall and Beedles (1994) measured both cytotoxicity and colony-forming potential of ES cells after 7 days of culture in the presence of test chemicals. In all of these *in vitro* tests with permanent ES cell lines the embryotoxic potential of only a few test chemicals was predicted correctly. This is most probably due to the fact that in the assays usually only two endpoints were determined, which is insufficient to assess essential mechanisms of embryotoxicity in mammals (Wilson and Frazier, 1977): inhibition of differentiation and differences in sensitivity between adult and embryonic tissues. Moreover, the timing for determining cytotoxicity and differentiation of ES cells may have to be optimized since cytotoxicity was usually measured after 24 h of culture and differentiation of ES cells after 7–10 days.

To overcome the limitations of the described

ES cell tests, we determined the following three endpoints, cytotoxicity both in 3T3 fibroblasts and ES cells (line D3) in the MTT test at different time points up to 10 days and inhibition of differentiation of ES cells into myoblasts on day 10 of culture. In the present study, we report the development of the EST, which was evaluated with 16 chemicals selected from a list recommended by the US Teratology Society for *in vitro* embryotoxicity test validation (Smith et al., 1983). In addition, the most predictive endpoints of the EST to identify embryotoxic chemicals were determined by stepwise discriminant analysis. These were then used in linear discriminant analysis to develop a classification procedure, which provided a correct classification of all of the 16 chemicals tested in the EST.

MATERIALS AND METHODS

Cell Culture Conditions

BALB/c 3T3 cells, clone 31, were purchased from ICN-Flow (Eschwege, Germany) and cultured as described previously (Spielmann et al., 1993). The mouse ES-cell line D3 (kindly provided by Dr. Kemler, MPI, Freiburg, Germany) was cultured in Dulbecco's modified Eagle's medium (DME-medium) with a 4.5 g/L glucose and 3.7 g/L NaHCO₃ (GIBCO BRL, Germany) supplemented with 20% FCS (Boehringer Mannheim, Germany), 50 U/ml penicillin and 50 µg/ml streptomycin (GIBCO BRL, Germany), 1% nonessential amino acids (NAA) (GIBCO BRL, Germany), 0.1 mM β-mercaptoethanol (Sigma, Germany), and 2 mM glutamine (GIBCO BRL, Germany), and maintained in the undifferentiated state by the addition of recombinant LIF (1000 U/ml) (GIBCO BRL, Germany) or conditioned medium from the LIF-Chinese hamster ovary (CHO) cell line 8/24 720 LIFD. D3 ES cells were subcultured every 2–3 days, when they reached approximately 80% confluence and they were used for not more than 25 passages.

Determination of Cytotoxicity with 3T3 Cells and D3 ES Cells

3T3 fibroblasts and D3 ES cells were used in the exponentially growing phase. Cells sus-

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pended in culture medium (1×10^4 cells/ml) were seeded in a volume of 50 μ l/well into 96 well flat-bottomed tissue culture microtiter plates (Falcon, Germany) and incubated in an humidified atmosphere with 5% CO₂ at 37°C for 2 h. In addition, a volume of 150 μ l culture medium/well was added containing the appropriate dilution of a test chemical. Two columns for solvent control and six columns for test chemicals with six aliquots per column were used for each concentration of test chemicals per microtiter plate. After 3 and 5 days the culture medium was removed and subsequently the same concentration of test substance as used on day 1 was added to the microtiter wells.

On days 1, 3, and 10 the MTT cytotoxicity assay according to Mosman (1983) was carried out with the following modifications (Laschinski et al., 1991). In brief, 20 μ l of 5 mg/ml (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT, Sigma, Germany) in PBS was added into each microtiter well and incubated at 37°C in a humidified 5% CO₂ atmosphere for 2 h. After elimination of the supernatant, cells were incubated with 130 μ l MTT "desorb-mix-solution" containing 3.49% (vol/vol) of a 20% solution of SDS solved in aqua dest. and 96.51% (vol/vol) of propan-2-ol. After agitation for 15 min on a microtiter plate shaker (MTS 4, IKA-Labortechnik, Germany) the plates were transferred to an enzyme-linked immunosorbent assay (ELISA) reader (Model EL309, BIO-TEK, Technomara, Germany) using a wavelength of 570 nm and a reference wavelength of 630 nm.

In Vitro Differentiation of D3 ES Cells into Contracting Myocardial Cells

D3 ES cells were harvested by trypsin/EDTA (GIBCO, BRL, Cat. No. 45300-019) treatment to reach single cell suspension starting an experiment. Two ml reaction medium were added, the cells were resuspended and subsequently centrifuged (120 g, for 4 min). The cell culture technique, which allows ES cells of the mouse to differentiate into contracting myocardial cells, was developed by Wobus et al. (1991).

An adaptation of the method for the ES cell line D3 in our laboratory has been published in detail (Heuer et al., 1994b). To induce cell differentiation the hanging drop method was cho-

sen (Wobus et al., 1991). A stem cell suspension (20 μ l) (3.75×10^4 cells/ml) in culture medium (DME-medium with 4.5 g/L glucose and 3.7 g/L NaHCO₃ [GIBCO BRL, Germany] supplemented with 20% FCS (Boehringer Mannheim, Germany), 50 U/ml penicillin and 50 μ g/ml streptomycin [GIBCO BRL], 1% nonessential amino acids [NAA] [GIBCO BRL], 0.1 mM β -mercaptoethanol (Sigma, Germany), and 2 mM glutamine [GIBCO BRL]) were placed onto the inner side of the lids of 10 cm bacterial-grade Petri dishes (approximately 60–80 drops were placed per dish) filled with 5 ml phosphate-buffered saline (PBS). After cultivation at 37°C in a humidified atmosphere containing 5% CO₂ for 3 days aggregates, termed embryoid bodies (EBs), were transferred into bacterial Petri dishes. After 2 days in suspension culture, one EB is placed into costar plates well (24 well flat-bottomed trays, Falcon 3047, Falcon Plastics, Germany) with further development into beating myocardial cells.

On day 10 of culture the beating myocardial cells were analyzed under an inverted microscope (Olympus, Hamburg, Germany) and the maximum percentage of this cell type in "costar plates" was determined by evaluation a total of 24 EBs. The control plates showed that 21 of 24 EBs differentiated into myocardial cells with contracting activity. To evaluate the toxicologic profile of test substances, an appropriate concentration of test chemicals was added into the lid of a bacterial Petri dish (day 1 of culture), into a bacterial Petri dish (day 3 of culture), and into a costar well (day 5 of culture) in a single test. Values of 50% inhibition of differentiation (ID₅₀) with D3 ES cells were determined graphically by plotting concentrations of test chemicals to the x-axis and the experimental values to the y-axis. ID₅₀ values for inhibition of differentiation were determined in percentage of values using the number of EBs with beating myocardial cells in control plates as 100% value.

Selection of Test Chemicals

Sixteen test chemicals with known embryotoxic potential were selected from a published list recommended by the US Teratology Society for the validation of *in vitro* embryotoxicity tests (Smith et al., 1983). The sixteen test chemicals, which in-

cluded four nonembryotoxic chemicals as controls, were assigned to three classes. Class 1 (four not embryotoxic chemicals): ascorbic acid, isoniazid, penicillin G; and saccharin. Class 2 (seven moderate embryotoxic chemicals): aspirin, caffeine, dexamethasone, diphenhydramine, diphenylhydantoin, indomethacin, and methotrexate. Class 3 (five strong embryotoxic chemicals): busulphan, cytosine arabinoside, 5-fluorouracil, hydroxyurea, and retinoic acid (RA). All test chemicals were purchased from Sigma (Germany) and dissolved as recommended by the supplier.

Statistical Analysis

Linear discriminant analysis (using the procedure "DISCRIM" of SAS/STAT, 1988) was used to identify the endpoints and/or ratios of endpoints of the EST that showed the best correlation to the embryotoxic potential of the compounds tested. In the three in vitro tests the following twelve experimental endpoints and ratios of endpoints were determined. The six endpoints of cytotoxicity were IC_{50} of 3T3 cells after 1, 3, and 10 days of culture; IC_{50} of ES cells after 1, 3, and 10 days of culture. The one endpoint of embryotoxicity was ID_{50} of ES cells after 10 days of culture (ID_{50} = 50% inhibition of differentiation). The five ratios of endpoints were IC_{50} 3T3 cells/ES cells after 1, 3, and 10 days of culture; IC_{50} 3T3 cells/ ID_{50} ES cells after 10 days of culture; and IC_{50} ES cells/ ID_{50} ES cells after 10 days of culture.

Since each of the endpoints and ratios of endpoints may contribute to classifying test chemicals successfully into one of the three classes of embryotoxicity, a stepwise selection of endpoints or ratios of endpoints was performed by applying discriminant analysis (procedure: "Stepdisc" of SAS/STAT, 1988). The procedure identifies the best endpoint to discriminate between the selected classes of embryotoxicity. Then, stepwise, each of the endpoints or ratios of endpoints is separately included in the model and thereafter rejected from the model if it does not improve the separation of the three classes of embryotoxicity. Inclusion or rejection of an endpoint is based on analysis of covariance, where an endpoint may be accepted, if the probability of error of the F-value is $\leq 15\%$ ($p \leq 0.15$). Practical experience with the present data set revealed that the pre-

diction was significantly improved by inclusion of an additional endpoint if the probability error was $\leq 5\%$ ($p \leq 0.05$). In addition, arithmetic and geometric mean values of ratios of endpoints are included in the "Stepdisc" procedure.

According to the sample size of chemicals represented in different classes, linear discriminant analysis was performed with proportional a priori probability. The classification of chemicals into one of the three classes of embryotoxicity obtained with *resubstitution* of the data into the linear prediction model was confirmed by the method of *cross-validation*, which allows one to predict the probability of correct and false classifications for future data sets. In the *cross-validation* procedure each chemical out of a group of "n" chemicals is classified into a model created with the remaining n-1 chemicals. The procedure is repeated n times with each of the chemicals and the remaining n-1 chemicals serve as a training set for the prediction model.

RESULTS AND DISCUSSION

Cytotoxicity on Days 1, 3, and 10 of Culture in 3T3 and ES Cells

To develop the EST, cytotoxicity of the 16 test chemicals was determined with 3T3 cells and D3 ES cells. Culture periods from 1 to 10 days were used to identify the most sensitive in vitro endpoint for predicting the embryotoxic potential. Cytotoxicity was determined in the MTT test, since we have previously shown that this assay provides more reproducible data with ES cells than other cytotoxicity tests, such as the neutral red uptake test and the Kenacid blue test (Laschinski et al., 1991). The MTT test was performed on day 1, 3, or 10 of culture in at least two independent experiments. The results of cytotoxicity testing are given in Table 1.

We have previously suggested comparing cytotoxicity data of adult fibroblasts (e.g. 3T3 cells) and of ES cells after exposure to test chemicals for 24 h in order to identify embryotoxic test chemicals (Laschinski et al., 1991). The data in Table 1 show that after 24 h of exposure the IC_{50} values of ES cells of the four nonembryotoxic chemicals of class 1 are almost identical to the IC_{50} values of 3T3 cells. In classes 2 and

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TABLE 1. CYTOTOXICITY TESTING AND INHIBITION OF DIFFERENTIATION

In vivo classification of test chemicals	3T3-cells			Cytotoxicity (MTT-test) ^a				ES-cells		Differentiation ^b ES-cells
	Days in culture 1	3	10	1	3	10	10			
Class 1: Not embryotoxic chemicals										
Ascorbic acid	170	115	25.5	227.5	240	138	408			
Isoniazid	2750	3275	375	1900	1675	750	360			
Penicillin G	6350	1650	1586	9400	8600	2950	3450			
Saccharin	9000	7500	3000	10000	5500	3498	2000			
Class 2: Moderate embryotoxic chemicals										
Aspirin	2100	310	230	1000	620	220	248			
Caffeine	795	310	155	283.3	228	165	185			
Dexamethasone	935	255	26	74	31	23	18.3			
Diphenhydramine	155	124	30	46	25	29.5	6.7			
Diphenylhydantoin	247.5	120	35	145	77	27.3	20			
Indomethacin	1300	105	27	312.5	73	29	66			
Methotrexate	54	0.027	0.015	30.5	0.032	0.074	0.020			
Class 3: Strong embryotoxic chemicals										
Busulphan	495	70.3	4.8	190.0	6.43	2.1	4.6			
Cytosine arabinoside	260	0.17	0.033	0.27	0.038	0.024	0.029			
5-Fluorouracil	800	0.29	0.17	0.19	0.073	0.103	0.029			
Hydroxyurea	355	14.8	7.2	16.3	3.1	2.0	1.7			
Retinoic acid	34.7	7.9	1.0	30.0	0.038	0.005	0.000105			

^aMean IC₅₀ values (μg/ml) of at least three determinations.^bMean ID₅₀ values (μg/ml) of at least three determinations.

3 (moderate and strong embryotoxic chemicals), however, all of the IC_{50} -values at 24 h are significantly lower for ES cells than for 3T3 cells, with the exception of retinoic acid (RA) in class 3. This result basically supports our earlier findings (Laschinski et al., 1991), but it was unacceptable for RA.

Taking into account that differentiation of ES cells in culture takes 10 days, 3T3 and ES cells were exposed up to 10 days to test chemicals, which were added to the cultures after change of media on days 3 and 5, respectively, in the same manner as in ES cell cultures. Table 1 also gives the cytotoxicity data obtained on days 3 and 10. IC_{50} values of 3T3 and ES cells are almost identical on days 3 and 10 for class 1 and 2 chemicals (not and moderate embryotoxic). The situation is more complex for chemicals of class 3 (strong embryotoxic), since on day 3 IC_{50} values of ES cells are one order of magnitude lower than IC_{50} values of 3T3 cells and on day 10 there is no obvious difference between IC_{50} values of 3T3 and ES cells for most of the chemicals with the exception of hydroxyurea and RA.

RA seems to be exceptional since it provides the lowest IC_{50} value of all chemicals tested on day 10 and also the biggest differences between IC_{50} values of 3T3 cells and ES cells on days 3 and 10. Since it is well known that RA is an endogenous factor influencing growth and differentiation of cells by interacting with specific RA receptors (Giguere et al., 1987; Petkovich et al., 1987), the result obtained with the two cell lines in cytotoxicity testing may not be that unexpected. The effects of exposure to RA could most probably not be detected after 24 h of culture on day 1 since RAR receptors of the cells were still saturated with RA. On days 3 and 10, however, the endogenous stores of RA were depleted and the effects of exogenous RA concentrations in the culture medium could be determined. This example shows that even in cytotoxicity testing, which seems very simple and straightforward, kinetics and metabolism have to be taken into account to draw valid conclusions.

Inhibition of Differentiation ES Cells

Since the results from cytotoxicity testing did not provide sufficient information for assessing

the embryotoxic potential of the chemicals tested in the present study, we tested their effect on the differentiation of ES cells *in vitro* in a second test. In this test the ability of ES cells to differentiate into several embryonic cell types is taken into account if leukemia inhibiting factor (LIF), a factor that inhibits differentiation of ES cells, is removed from the culture medium. We have reported in earlier studies (Heuer et al., 1994a,b) that in a very reproducible manner ES cells of the D3 line will differentiate during a 10 day culture period into contracting myocardial cells.

Inhibition of ES cell differentiation is also shown in Table 1. ID_{50} values obtained with chemicals of classes 1–3 of embryotoxicity clearly show a ranking that correlates well with the embryotoxic potential of the test chemicals in the following order: ID_{50} values class 1 > ID_{50} class 2 > ID_{50} class 3. A more critical evaluation shows that the ranking is not perfect for chemicals of classes 2 and 3 (moderately and strongly embryotoxic). For some of the chemicals of class 2 (e.g. diphenpyramine and methotrexate) ID_{50} values were obtained that are similar to ID_{50} values measured of class 3 chemicals (e.g., busulphane and hydroxyurea).

From the results of testing the 16 test chemicals in the ES cell differentiation test one must conclude that the test does not provide sufficient information to predict correctly the embryotoxic properties of test chemicals. This result is, again, not unexpected since according to present knowledge embryotoxicity induced by exposure to chemicals in mammalian systems is in most instances not caused by a single mechanism (e.g., inhibition of differentiation at a critical period of development) but also by growth inhibition of cells dividing at a very high rate (Wilson and Frazier, 1977). In order to take as much of the information on differentiation of ES cells and on growth inhibition of ES cells and of 3T3 cells into account, we used a biostatistical approach to optimize the EST.

Statistical Analysis and Development of a Classification Model

From the data of the three *in vitro* tests, the seven endpoints and five ratios of endpoints de-

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scribed in the Materials and Methods section were selected to develop the best prediction model to classify chemicals according to their embryotoxic potential. The means of the endpoints were entered into and analyzed by the procedure of stepwise linear discriminant analysis (= "step disc" procedure of SAS/STAT, 1988). Variability of the endpoints was not taken into account during discriminant analysis.

From the 12 endpoints and ratios of the EST the following three endpoints and ratios were selected since they showed the best correlation with the embryotoxic potential of the test chemicals: **lgES**: logarithm of IC50 of ES cells on day 10; **lg3T3**: logarithm of IC50 of 3T3 cells on day 10; **R**: ratio IC50 of ES cells/ID50 of ES cells both on day 10.

In the next step the three endpoints identified as strongly discriminating to identify embryotoxic chemicals in the test set of 16 of the present investigation were entered into the procedure "discrim" for linear discriminant analysis (DISCRIM of SAS/STAT, 1988). Using the three endpoints identified by the step disc procedure, two linear discriminant functions were selected by linear discriminant analysis to classify chemicals into the classes 1-3 of embryotoxicity defined above.

Applying the two linear discriminate functions to classification of chemicals into the three classes of embryotoxicity, the following procedure was used:

In step 1 a chemical is assigned to class 1 (= not embryotoxic), if criteria of the following discriminant function are fulfilled:

$$91.9 \lg ES - 70.2 \lg 3T3 + 4.9 R - 49.1 \\ > 40.7 \lg ES - 31.1 \lg 3T3 + 2.2 R - 10.3$$

If not, step 2 follows, in which the chemical is assigned to class 2 (moderate embryotoxic) if

$$40.7 \lg ES - 331.1 \lg 3T3 + 2.2 R - 10.3 \\ > -26.3 \lg ES + 20.5 \lg 3T3 - 1.3 R - 5.3$$

If not, step 3 follows: the chemical is then assigned to class 3 (strong embryotoxic).

The classification model, which takes into account only cytotoxicity data (IC₅₀) of ES cells

and of 3T3 cells on day 10 and the inhibition of differentiation of ES cells on day 10 (ID₅₀), provided the result shown in Table 2. Quite unexpectedly the classification model provided a 100% correct classification of the 16 test chemicals into the 3 *in vivo* classes of embryotoxicity. This result was confirmed by two types of statistical validation: resubstitution and cross-validation.

To further test the classification model cytotoxicity data obtained with 3T3 cells on day 1 (after 24 h) and day 3 were used in the classification model instead of day 10 data. The results of classification were less sufficient than with the model using day 10 data. Thus the classification model developed for the ETS seems to be very robust.

Further Development and Validation of the EST

The new EST was developed with two permanent mouse cell lines: the ES cell line D3 and 3T3 fibroblasts. Thus in the EST, in contrast to most of the other *in vitro* embryotoxicity tests, no pregnant animals have to be sacrificed for testing. Compared to other cellular *in vitro* embryotoxicity tests, two important aspects of prenatal toxicity have been taken into account: inhibition of differentiation of embryonic cells (the

TABLE 2. *IN VITRO* CLASSIFICATION OF TEST CHEMICALS IN THE EST

	Class ^a	In vitro classification		
		1	2	3
<i>In vivo</i> activity	1	4	0	0
	2	0	7	0
	3	0	0	5

^aSixteen test chemicals were assigned to three *in vivo* classes: class 1, not embryotoxic: four chemicals; class 2, moderate embryotoxic: seven chemicals; class 3, strong embryotoxic: five chemicals.

Linear discriminant functions were used for classifying test chemicals into three *in vitro* classes. Results were identical when resubstitution or cross-validation were used in the classification procedure.

ES cells), and also the comparison of differences in sensitivity of embryonic cells, again ES cells, and "adult" cells, mouse fibroblasts of the cell line 3T3, in the MTT cytotoxicity test. A similar approach was used by Newall and Beedles (1994) to develop a stem cell test for *in vitro* embryotoxicity testing, in which only ES cells were used and the two endpoints cytotoxicity and colony formation. The first results reported with their stem cell test were not as convincing as the results obtained in the EST in the present study. The two endpoints used by Newall and Beedles (1994) in their stem cell test may not have been sufficient for embryotoxicity testing since a difference in sensitivity between adult and embryonic tissues, which is essential information to evaluate the embryotoxic potential of chemicals, is not provided by their assay.

However, the approach used by Newall and Beedles (1994) to assess the inhibition of differentiation of ES cells chemicals has some advantage for routine testing compared to our EST. Colony formation in their assays is determined automatically, while the quantification of contracting myocardial cells in ES cell cultures is difficult to standardize and requires well-trained and experienced technicians. We are, therefore, trying to use monoclonal antibodies to label myosin in differentiating ES cells in order to develop an automatic procedure to monitor differentiation of ES cells. Since ES cells will differentiate under appropriate conditions into other tissues than myocardial and since myocardium is not the prime target of embryotoxic agents in humans, differentiation of ES cells into other tissues (e.g., cartilage and nerve tissue) will be incorporated into the EST.

So far no other *in vitro* embryotoxicity test has shown a better performance than the EST in the present study (Schmid, 1985; Brown et al., 1995). The EST should, therefore, be evaluated in other laboratories and more chemicals should be tested in the new test. A major problem for using ES cells for testing under routine laboratory conditions resulted from difficulties in handling and maintaining ES cells, which tend to differentiate spontaneously. To overcome this problem, we developed a standard protocol (SOP) that is currently being evaluated for its robustness in an ECVAM program on prevalidation (Curren et al., 1995). A recent ECVAM/ETS workshop on meth-

ods for screening chemicals for reproductive toxicity (Brown et al., 1995) has strongly recommended making better use of ES cells for *in vitro* embryotoxicity testing.

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Address reprint requests to:
 Horst Spielmann, M.D.
 Head of ZEBET at the BgVV
 Diedersdorfer Weg 1
 D-12277 Berlin, Germany